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The evolution of host specialization in an insect pathogen

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Niche breadth coevolution between biotic partners underpins theories of diversity and co-existence and influences patterns of disease emergence and transmission in host-parasite systems. Despite these broad implications, we still do not fully understand how the breadth of parasites' infectivity evolves, the nature of any associated costs, or the genetic basis of specialization. Here, we serially passage a granulosis virus on multiple inbred populations of its *Plodia interpunctella* host to explore the dynamics and outcomes of specialization. In particular, we collect time series of phenotypic and genetic data to explore the dynamics of host genotype specialization throughout the course of experimental evolution and examine two fitness components. We find that the *Plodia interpunctella* granulosis virus consistently evolves and increases in overall specialization, but that our two fitness components evolve independently such that lines can specialize in productivity or infectivity. Furthermore, we find that specialization in our experiment is a highly polygenic trait best explained by a combination of evolutionary mechanisms. These results are important for understanding the evolution of specialization in host-parasite interactions and its broader implications for co-existence, diversification, and infectious disease management.

KEY WORDS: **Experimental evolution, host range, host-parasite interactions, specialization.**

The question of why some species are specialists and others are generalists has been central to evolutionary biology since its inception (Darwin 1859). This co-existence of strategies is commonly explained by there being some cost to generalism such that specialists are favored under certain ecological conditions (Futuyma and Moreno 1988) because "jacks-of-all-trades are the masters of none" (MacArthur 1984). The theory of costly generalism has been extensively applied in the host-parasite eco-evolutionary literature to explain parasite niche breadth and specialization at the levels of both host species and host genotype (Regoes et al. 2000; Gandon and Poulin 2004; Osnas and Dobson 2012). Niche breadth at the level of host species has important implications for pathogen emergence (Guth et al. 2019) and species co-existence (Janzen 1970; Connell 1971); while niche breadth and specialization at the genotype level underpins the monoculture effect (Elton 1958), local adaptation (Kawecki and Ebert 2004), and the Red Queen Hypothesis of Sex (Jaenike 1978).

Despite the broad implications of niche breadth evolution in antagonistic coevolutionary systems, there is still debate about whether costs to niche breadth are, in fact, universal and what the dominant genetic mechanisms driving such costs would be (Jaenike 1990; Remold 2012). Several mechanisms for the evolution of specialization have been proposed. The classic

trade-off hypothesis expects that increases in fitness on one host negatively trade-off with fitness on foreign hosts (Levins et al. 1968; Regoes et al. 2000). These strict negative trade-offs are not universal though, so several additional theories have been proposed including host specialization due to weakly positive or neutral genetic correlations leading to asymmetrical fitness gains (Fry 1996) and host specialization due to the accumulation of deleterious mutations on alternate hosts (Kawecki 1994; Whitlock 1996). The number of genes involved in specialization could also vary so that it is driven by a few mutations of large effect or by many mutations of small effect.

Experimental evolution approaches have been used to explore the evolution of specialism and the nature of costs to generalism in a wide range of taxa evolving to a variety of selective environments (Cooper and Lenski 2000; Kassen 2002; Bono et al. 2017; Visher and Boots 2020). Host genotype specialization, specifically, has been studied with great success in host-parasite systems including mice and RNA virus (Kubinak et al. 2012), mosquitos and microsporidia (Legros and Koella 2010), daphnia and bacteria (Little et al. 2006), protists and bacteria (Nidelet and Kaltz 2007), *C. elegans* and bacteria (Schulte et al. 2011), and wheat and fungus (Zhan et al. 2002). Generally, these studies find that serial passage on a single host genotype increases fitness on that host genotype while decreasing or at least resulting in smaller fitness gains on other genotypes.

However, there has been a limited empirical exploration of the genetic mechanisms of such specialism. Similar work has explored the genetics of virus specialization to different host cell lines, finding that specialization was driven by a mix of antagonistic pleiotropy and mutation accumulation depending on a lineage's evolutionary history (Remold et al. 2008), but this inquiry has not yet been extended to specialization to different host genotypes. A better understanding of the genetics of specialization is important because the number of potential mutations involved in host genotype specialization and the genetic mechanism of such specialization will affect the evolutionary dynamics of specialization and may create divergent predictions for eco-evolutionary theory (Remold 2012; Visher and Boots 2020).

In this paper, we explore the evolutionary dynamics of host genotype specialization in the *Plodia interpunctella* (Hübner) and *Plodia interpunctella* granulosis virus (PiGV) laboratory model system. *Plodia interpunctella*, the Indian meal moth, is a stored grain pest that has been extensively used to characterize trade-offs and test eco-evolutionary dynamics in the lab (Boots and Mealor 2007; Boots 2011; Bartlett et al. 2020, Bartlett et al. 2018). We experimentally evolve virus populations to determine whether PiGV evolves to specialize on familiar host genotypes, collect multiple fitness metrics at multiple time points to explore the phenotypic dynamics of specialization, and sequence virus populations at multiple time points to explore the genetic mechanisms of specialization. We find that serially passaging virus leads to consistent increases in the specialization of familiar host genotypes through the course of experimental evolution, and that specialization can occur in multiple fitness components. MCMC-based inference analysis of time series data shows that this specialization is not driven by a few mutations of large effect (Schraiber et al. 2016). Combining these lines of evidence suggests that a combination of genetic mechanisms is likely to explain specialization in our system.

Methods

STUDY SYSTEM

Our study system is *Plodia interpunctella* (Hübner), the Indian meal moth, and the Plodia interpunctella granulosis virus (PiGV). *Plodia interpunctella* is a pest that lives in grain stores (Mohandass et al. 2007). During its five larval instar stages, it develops within its food medium before pupating and emerging into an adult moth. For this experiment, we use inbred lines previously generated in Bartlett et al. (2018). These lines were made by mating individual brother-sister pairs for more than 27 generations. At this point, inbred populations should represent near-clonal populations of a single genotype that was randomly selected from the genetically diverse founder population via drift. Limited data suggest that these inbred lines had levels of resistance similar to other selection lines in our lab (Bartlett et al. 2020), though it is possible that inbreeding could have affected resistance quality. However, we would not expect this to alter our characterizations of the dynamics of specialization since all our specialization metrics are relative across equally inbred lines.

Plodia interpunctella granulosis virus (PiGV) is a dsDNA baculovirus that is an obligate killer (Vail and Tebbets 1990). The natural life cycle is as follows: a larvae ingests virions in the occlusion body form, the virions shed their protein coats and infect gut epithelial cells, the virions either pass through the gut to establish a successful infection or are cleared during molting (freeing the larvae to carry out the rest of their life history), the virus begins to proliferate through the entire body of the larvae, and, once at a critical mass, packages into the protein-coated occlusion body form and kills its host (Rohrmann 2013). It can then be transmitted to susceptible larvae when they cannibalize infected cadavers and ingest occluded virus. Critically, the virus must kill its host in order to transmit, and larvae can only pupate and become adult moths if they were not successfully infected (Boots and Begon 1993).

HOST SELECTION AND MAINTENANCE

We selected three inbred *Plodia interpunctella* populations with similar overall levels of resistance for this experiment, as

measured by a preliminary resistance assay of all twelve of the inbred populations (Table S1). The chosen inbred populations (Lines 2, 9, and 17) represent genotypes with similar medium overall levels of resistance compared to the full set of potential inbred lines (Table S1). Populations of these genotypes were maintained in the absence of the virus as in (Bartlett et al. 2020) (See Supplemental Methods for details).

SETTING UP EXPERIMENTAL EVOLUTION

Virus evolution was initiated with a single genetically diverse virus stock that we diluted to a passaging dose that would cause high mortality ($\sim 7.5 \times 10\hat{}8$ occlusion bodies per mL). A 0.5mg early third instar larvae eats <0.1mg of the solution (unpublished data), corresponding to an exposure dose of <75,000 occlusion bodies. This value is consistent with natural field doses of baculovirus, which tend to be very high (Eakin et al. 2015; Kennedy and Dwyer 2018).

We counted the concentration of this passaging dose on a Petroff-Hauser counting chamber with a darkfield microscope at 400x magnification. This dilution was combined with 2% sucrose (ThermoFisher Scientific, U.S.A.) and 0.2% Coomassie Brilliant Blue R-250 dye (ThermoFisher Scientific, U.S.A.). The sucrose encourages the larvae to consume the virus solution and the dye allows us to recognize larvae that have consumed half their body length of virus solution and are therefore considered successfully inoculated.

We set up three replicate evolving lines of virus on each of the three inbred host genotypes (see Fig. S1 for passaging scheme). For each virus line, we collected 100 third instar larvae of the appropriate genotype in a petri dish and starved them under a damp paper towel for 2 hours. We then syringed tiny droplets of our virus-sucrose-dye solution onto the petri dish for the larvae to orally ingest. After about an hour, we moved 50 successfully inoculated larvae into two 25-cell compartmentalized square Petri dishes (ThermoFisher Scientific, U.S.A.) with standard food. The grid plates were then transferred to a single incubator for 20 days.

SERIAL PASSAGE

After 20 days, we harvested virus from each virus line under sterile conditions by collecting up to 10 virus killed cadavers per line and transferring these to sterile 15 mL disposable tissue grinders (ThermoFisher Scientific, U.S.A.). Infected larvae were recognizable by their opaque, chalky, white coloration. We were not able to collect 10 infected cadavers from all virus lines at all passages, so, when we could not find 10 infected cadavers, we collected every infected cadaver that we could find (Table S2). To extract virus from infected cadavers, we added 2mL of sterile DI water to the tissue grinders and homogenized the solution until all cadavers had been thoroughly crushed. We then transferred 1mL of the supernatant to a sterile 1.5mL Eppendorf tube and centrifuged the solution for 1 minute at 3000 rpm to remove larger particulate matter from the supernatant. We transferred 600uL of this solution to a sterile 1.5 mL Eppendorf and centrifuged this for 3 minutes at 13,000 rpm to pellet the virus. We removed the supernatant from the pellet and resuspended in 1mL sterile water.

After extracting the virus, we diluted the solution 10× and added 600 μL of the dilution to a .65 micron filter spin column (Millipore Sigma, USA) that we centrifuged at 13,000 rpm for 3 minutes to semi-purify the virus of possible bacterial and fungal contaminants (for method details see Table S3). Importantly for later comparisons, this purification method differed from the sucrose gradient purification method used to generate the ancestral virus stock (Harrison et al. 2016) and may have resulted in differences in infectivity per particle. We counted each of the semi-purified virus solutions as above and diluted them to the passaging dose concentration of $\sim 7.5 \times 10^8$ occlusion bodies per mL in 2% sucrose and .2% dye to form our final passaging solutions for each virus line. A portion of these virus dilutions were then used to infect the next set of third instar larvae of the appropriate genotype following the protocol above and the rest was stored at −20C for assays and sequencing. Virus was serially passaged for nine passages (Figure S1). The number of passages was determined at the start of the experiment and was based on numbers standard for similar experiments (Zhan et al. 2002; Nidelet and Kaltz 2007; Legros and Koella 2010; Kubinak et al. 2012).

ASSAYING

We assayed each virus line at multiple passages to track evolution over the course of the experiment (Figure S1). We assayed the starting population of virus as well as virus harvested from passages 1, 4, 6, and the final passage 9. For each assay, we inoculated all 3 host genotypes with all nine virus lines at both the passaging dose and 10% of the passaging dose. We inoculated 25 larvae for each host genotype × virus line × dose combination using the standard inoculation protocol above. Because of time constraints, inoculations for each passage were conducted across three days with one host genotype each day being inoculated with all of the virus lines. By assaying all the virus populations from each of the evolutionary histories on all of the host genotypes, we were able to measure how the evolving virus line changed in fitness on the familiar (the genotype that the virus evolved on) and foreign (genotypes that the virus was unexposed to) host genotypes.

After 20 days, we froze the grid plates and counted the number of infected and uninfected individuals in each grid. This proportion infected is our viral 'infectivity' metric. We collected all the infected larvae from each assay grid that had been inoculated with the higher dose and froze them in a pooled sample per grid plate. We extracted virus from these samples via tissue grinding and the two centrifugation steps (without filtering) and counted

the virus in a Petroff-Hauser counting chamber as above. From these virus counts and the number of infected larvae, we were able to determine how many occlusion bodies each virus line produced per infected cadaver on average when infecting each host genotype at the high dose. This average number of occlusion bodies per infected cadaver at the high dose is our viral 'productivity' metric.

Finally, we multiplied the average number of virions produced per infected cadaver by the proportion of larvae infected to get a composite measure of fitness for each virus line on each host genotype at the high dose. This is hereto after referred to as 'fitness'.

SEQUENCING AND VARIANT CALLING

The ancestral virus population and virus populations for each line at the four assayed time points (37 samples) were next prepared for sequencing. First, extracted occlusion bodies were rinsed in 0.1% SDS and purified in a Percoll gradient as in (Gilbert et al. 2014). Occlusion bodies were then dissolved in 0.5M Na_2CO_3 and DNA was extracted with a QIAamp DNA kit. Library preparation and sequencing were conducted at the UC Berkeley QB3 center on non-amplified DNA. 150 bp paired-end libraries were generated with Kapa Biosystems library preparation kits and multiplexed to run on one lane of an Illumina MiSeq platform. Reads were then de-multiplexed and aligned to the PiGV reference genome [GenBank: KX151395] using bowtie2 (Langmead and Salzberg 2012; Harrison et al. 2016). The resulting alignments for each sample had 99.99-100% genome coverage, 51–100 mean coverage depth, and 40.2-41 mean MapQ scores. The ancestral population .bam file was then re-aligned to the reference, indel and alignment quality scores were added (dindel method), and variants were called (SNV and indel, minimum coverage = 20, default parameters) using LoFreq (Version 2.1.5) (Wilm et al. 2012) in usegalaxy.org (Afgan et al. 2018). LoFreq filter was used to select variants above 0.5 frequency to create a new consensus fafsa file using bcftools consensus (Version 1.10). FastQ files from all samples were then realigned to this consensus using bowtie2 (Version 2.4.2) and Samtools (Version 1.13) (Li et al. 2009) and variants were called using LoFreq as above. Variants were then filtered using LoFreq filter to select those above 0.01 frequency. The Galaxy history can be viewed here: https://usegalaxy.org/u/evisher/h/reviews2022final.

PHENOTYPIC ASSAY DATA ANALYSES

We analyzed all phenotypic assay data using a linear mixed modeling approach in R (version 4.0.3) using packages 'lme4'(Bates et al. 2015) and 'glmmTMB' (Brooks et al. 2017) to build models, 'DHARMa' (Hartig and Lohse 2021) to check residuals, 'afex' (Singmann et al. 2019) and 'car' (Fox and Weisberg 2019) to check model effects, 'emmeans' (Lenth 2019) to extract effects, and 'tidyverse' (Wickham et al. 2019) to manipulate data. Our response variables were either fitness, infectivity, or productivity of the virus line. Error structures for models were determined by testing model residuals with 'DHARMa' and then adjusting error structures to best normalize the residuals. We corrected residual distributions by sequentially testing models with observation level random effects (Harrison 2014), negative binomial distributions, then zero-inflated negative binomial or quasi-Poisson distributions as needed (See annotated R code).

The first part of our analysis looked at data from the end of the evolution experiment (passage 9). We tested for an effect of specialization by using a "self" factor that was either true (virus was assayed on the same host genotype it was evolved on) or false (virus was assayed on a host genotype it was not evolved on). We included this as a fixed effect alongside "assay genotype" and "evolution genotype" (the host genotype used for the assay and that the virus was evolved on, respectively). In the case of the 'infectivity' data analysis, "dose" was also included as a fixed effect. Our random effects were "evolution genotype" and "virus line," with "virus line" nested under "evolution genotype" to account for our experimental structure. Our infectivity model used a binomial error structure and our productivity and fitness models used Poisson error structures with observation-level random effects. To see if there were differences in which fitness metrics the "evolution genotypes" specialized on, we built "fitness," "infectivity," and "productivity" models specified the same as above, but with "self" only included as in interaction term with "evolution genotype." To see if there were differences in the ability of each virus selection line to evolve any specialism, we further analyzed the effect of "self" on fitness by including it as an interaction effect with "virus line" in a model specified the same as above, but with "virus line" replacing "evolution genotype" as a fixed effect and a negative binomial error structure. All model tables are provided in the Supporting Information Model Tables file and organized by test. For full model structure, see Supporting Information Model Tables M1.1-M1.7.

We also analyzed our infectivity, productivity, and fitness data across the whole experiment, including passages 1, 4, 6, and 9 to interrogate how specialization evolved with time. We did not include passage 0 data in this analysis because of clear differences in passage 0 to 1 fitness (likely due to different virus storage and extraction conditions) and differences in the underlying data structure of passage 0 data compared to evolved passage data (due to ancestral virus not yet being "split" into virus lines). We used the same general approach as detailed above, where fixed effects were "assay genotype," "evolution genotype," "self," "passage number," and an interaction between "passage number" and "self." Our error structure included "evolution genotype," "virus line" and "passage number," with "virus line" nested under "evolution genotype" as above, and "passage number" nested under

"virus line" to account for multiple generations acting as repeated measures. Our infectivity model used a binomial error structure with observation level random effects and our productivity and fitness models used zero-inflated negative binomial error structures. For full model structure, see Supporting Information Model Tables M2.1-2.3.

We further used a similar modeling approach to test for correlations between virus fitness on familiar and foreign hosts across the whole experiment by building a model for "fitness" including the interacting fixed effects of "assay genotype" and "passage number" and the same "passage number," "virus line," "evolution genotype" nested error structure as above and then extracting the residuals for each measurement of fitness of each virus line on each assay genotype. These residuals were used to build a "fitness on familiar genotypes" and "average fitness on foreign genotypes" dataset that we used to test whether "fitness on familiar hosts" was predicted by "average fitness on foreign hosts" and whether this effect interacted with the "evolution genotype." We further used the same modeling approach to test for a correlation between a virus line's virion production and its infectivity by including the proportion infected as an additional fixed predictor in a separate model of viral productivity at the highest dose. For full model structure, see Supporting Information Model Tables M3.1-M3.5.

We used the "ggplot2" (Wickham 2009) and "patchwork" (Pedersen 2020) packages to plot graphs of our results. See Supporting Information for annotated code.

VARIANT ANALYSIS

Variant frequencies were analyzed to: (1) identify genetic regions of variation in our population, (2) determine whether variant community composition was predicted by treatment, and (3) identify signatures of positive selection across the time series.

To determine regions of variation, we plotted variant frequencies against genome position, identified genome regions with high genetic variation, and compared these genetic regions to the annotated PiGV reference genome by hand to identify potentially interesting nearby genes (Harrison et al. 2016). To determine whether passage 9 variant community composition was predicted by treatment, we made multidimensional scaling plots in "vegan" using the "metaMDS" function with the 'Canberra' method, which deemphasizes zero values (Oksanen et al. 2020; Middlebrook et al. 2021). We then used constrained ordination analysis on Hellinger and Chi-square pre-transformed SNP frequencies in 'vegan' and performed a Monte Carlo permutation test to determine whether treatment significantly predicted SNP frequency variance amongst the virus populations (Oksanen et al. 2020). See Supporting Information for annotated code.

Finally, to identify signatures of positive selection, we used an MCMC-based inference procedure to infer the strength of selection acting at variable positions in our genomic time series data (Schraiber et al. 2016). This software estimates selection coefficients given an observed frequency trajectory, accounting for uncertainty in true allele frequencies due to binomial sampling. While we knew the average virus population size within a single individual at the end of infection, we did not know the exact number of virus particles that were found in each infection. However, we can apply functions derived from another baculovirus and lepidoptera system in Kennedy and Dwyer (2018) to estimate that infections are founded by about 37–42 virions as third instar larvae ingest <0.1mg of virus solution (unpublished data), corresponding to a likely exposure dose of ~7500–750,000 occlusion bodies. Thus, we chose several possible demographic models based upon a range of reasonable inoculums (from 35 to 200 viral particles) and a range of growth rates (including "slow" and "fast" exponential processes with ~1.2–5 fold growth per generation) and calculated the harmonic mean of population size and the number of generations needed to reach 10^{10} particles for each scenario (Harpak and Sella 2014). We then repeated our estimates of selection strength using each of these effective population sizes, which ranged from small to moderate (N_e = 92 to N_e = 2869). We call "significant" alleles using the most conservative demographic model (N_e = 92) by a loose threshold, where the 90% HPD interval did not overlap 0 (Figure S5). For details, see Supporting Information methods and annotated code.

Results

SPECIALIZATION OF VIRUSES AT THE FINAL PASSAGE

After nine passages of experimental evolution, we find good evidence that viruses evolved to specialize on their familiar host genotype, indicated by a significantly positive effect of "self" on viral infectivity (estimate = 0.33, $p = 0.014$, Fig. 1a and d, M1.1), productivity (estimate = 0.76, $p = 0.016$, Fig. 1b and e, M1.2), and fitness (estimate = 0.91, $p = 0.01$, Fig. 1c and f, M1.3; See Fig. 1, Supporting Information Model Tables M1.1-M1.3). Therefore, the evolved virus lines infected relatively higher proportions of individuals, produced more virions per infection, and therefore had higher fitness when infecting the host genotype that they had evolved on than when infecting foreign host genotypes.

We found a significant effect of "dose" ($p < 0.001$, M1.1) for infectivity, as expected, and a significant effect of "assay genotype" (host) for infectivity ($p < 0.001$, M1.1) but not productivity (M1.2) or fitness (M1.3). We did not find significant effects of "evolution genotype" on any of our three metrics (M1.1–M1.3), meaning that specific host genotypes did not lead to the evolution of generally more infectious or higher

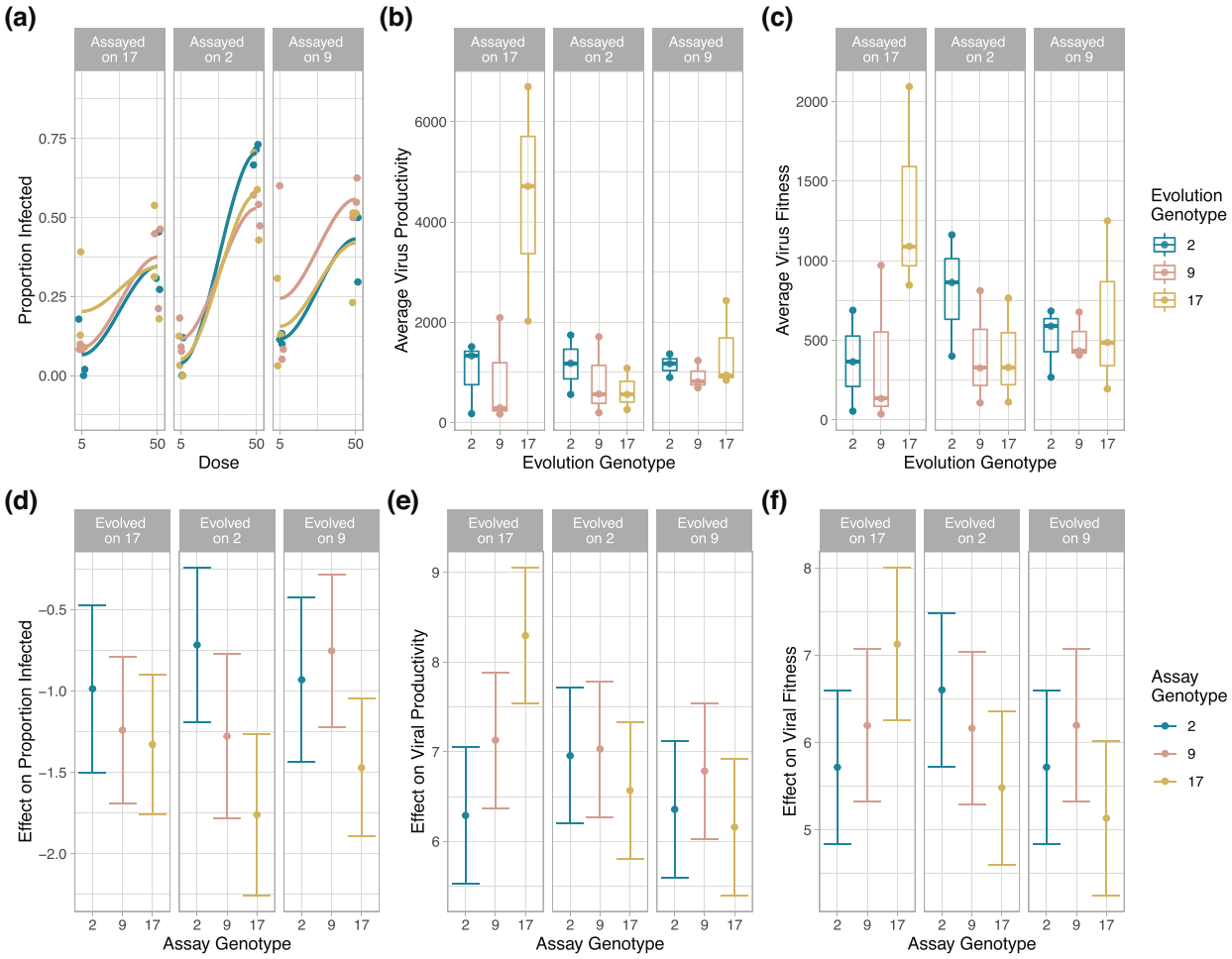

Figure 1. **Specialization of virus at the end of the experiment. Paneled plots show the effect of the virus's evolutionary history on its (a,d) infectivity, (b,e) productivity, and (c,f) composite fitness when infecting each of the assay lines. Panels on plots (a–c) are organized by the assay genotype as assays were conducted on different days. Panels on plots (d–f) are organized by the evolution genotype, as this better matches our question of how virus lines evolved to specialize on their familiar host. Productivity metrics were only collected at the high dose. Fitness is the proportion infected at the high dose *x* the average number of virions produced per infected cadaver. Panels (a–c) present raw data while panels (d–f) present effect size estimates and errors from the GLMM models.**

fitness virus populations when averaged across all three assay genotypes.

Next, we asked whether different evolution treatments led to differences in the specialization of different fitness metrics. In our fitness model, we do see a significant interaction between "evolution genotype" and 'self' ($p = 0.03$, M1.5) driven by higher specialization of lines evolved on host genotype 17. We do not find a significant interaction in our infectivity models ($p = 0.104$, M1.6). We do see a significant ($p = 0.017$, M1.7) interaction in our productivity model, however, driven by higher productivity specialization of lines evolved on host genotype 17 (fitness: estimate = 1.91, $p = 0.002$). Finally, we test whether virus lines differ in their fitness and find that they have significant fitness differences ($p = 0.012$) and interaction effects with "self" ($p = 0.0028$). See Supporting Information Model Tables M1.1-1.7 for full models and results.

EVOLUTION OF SPECIALIZATION OVER TIME

Our analysis of fitness data across all evolved passages (1, 4, 6, and 9) showed significant effects of passage number ($p = 0.001$), evolution genotype ($p = 0.0006$), and assay genotype (0.035) on virus fitness (Fig. 2; Fig. S2, M2.1). Virus lines had significantly ($p = 0.025$) lower fitness when assayed on host genotype 17, while virus lines evolved on host genotype 17 were significantly more fit ($p = 0.034$; M2.1). There is a significant effect of passage number on virus fitness ($p = 0.001$), with virus lines generally increasing in their total fitness from passage 1 to passage 4 and no further meaningful change from passage 6 to 9

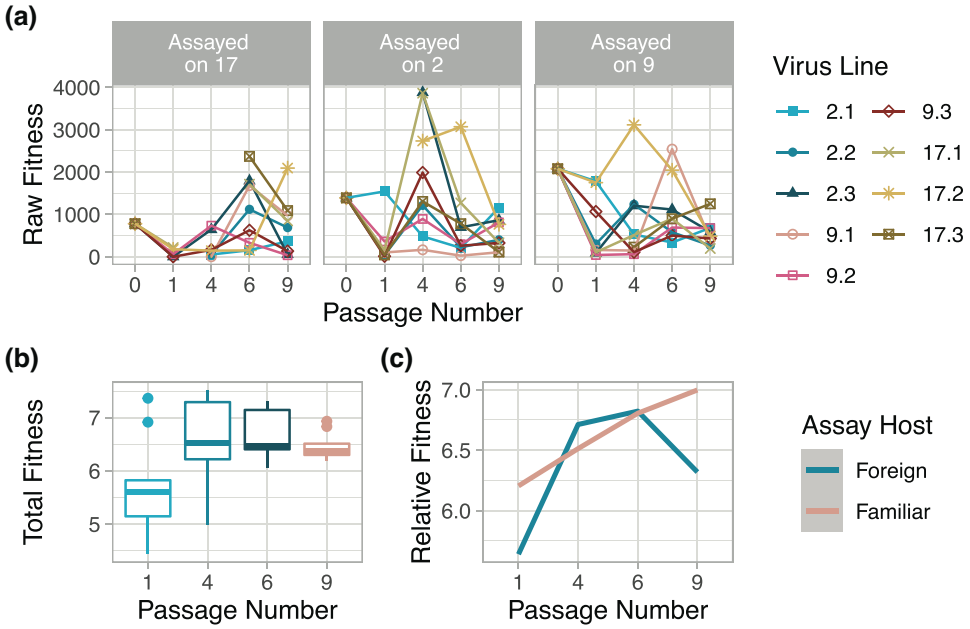

Figure 2. **Evolution of specialization over time. Paneled plot showing (a) raw data of each virus line's fitness on each assay line across the experiment, (b) the statistical effect of passage on total fitness across hosts, and (c) the statistical effect of whether the virus was assayed on its familiar host genotype (red) or on a foreign one (blue) on viral fitness over time. *Y*-axis effect sizes and errors for (b) and (c) are taken from the GLMM models using the "emmeans" package.**

(Pass4-1: estimate = 0.69, $p = 0.04$; Pass6-4: estimate = 0.2, $p = 1.0$; Pass9-6: estimate = −0.16, $p = 1.0$) (Fig. 2b, Fig. S2B, M2.1). There is not a significant interaction between the effect of infecting a familiar host and passage number ($p = 0.16$), and viruses only become significantly specialized at passage 9 (FALSE-TRUE: estimate = −0.68, $p = 0.03$; Fig. 2c, M2.1). This is because fitness on foreign hosts inconsistently changes (Pass4-1: estimate = 1.07, $p = 0.005$; Pass 6-4: estimate = 0.11, $p = 1.0$; Pass 9-6: estimate = −0.5, $p = 0.15$), even though fitness on familiar hosts has nonsignificant, consistent increases (Pass4-1: estimate = 0.31, $p = 1.0$; Pass 6-4: estimate = 0.29, $p = 1.0$; Pass 9-6: estimate = 0.19, $p = 1.0$; Fig. 2c).

CORRELATION BETWEEN FITNESS ON FAMILIAR AND FOREIGN HOSTS

We next determined the correlation between a virus line's fitness on their familiar host genotype and on the foreign host genotypes. A negative correlation would mean that the virus lines with the highest fitness on their familiar genotype had the lowest fitness on foreign genotypes and indicate a strict trade-off. Across the passage 1–9 dataset, we find that there is not a generally significant correlation between fitness on familiar and foreign hosts ($p = 0.13$, M3.1), nor does this relationship significantly change over time ($p = 0.738$; Fig. S3, M3.3). However, there is a significant interaction effect between the genotype that the lineage evolved on and the relationship between fitness on familiar and foreign hosts ($p = 0.001$, M3.1). Specifically, the relationship between fitness on familiar and foreign hosts is negative for lines evolved on genotype 17, positive for lines evolved on genotype 2 ($p = 0.002$), and not significant for lines evolved on genotype 9 ($p = 0.16$; Fig. 3, M3.1).

RELATIONSHIP BETWEEN VIRUS PRODUCTIVITY AND INFECTIVITY

When we examine the passage 9 dataset, we find that the relationship between virus productivity and infectivity significantly ($p = 0.007$, M3.5) interacts with whether the virus is infecting familiar or foreign hosts so that the relationship is negative when lines are assayed on their familiar genotype and positive when they are assayed on foreign. However, when we analyzed the full dataset with all passages, we do not find a significant three-way interaction between the effects of virus infectivity, virus productivity, and being assayed on the familiar genotype. Therefore, we fit and tested a model with an interaction effect between "self," "productivity," and "passage number" (M3.4). We do not find a generally significant interaction between these three metrics ($p = 0.067$, M3.4), but do find that the interaction between infecting a familiar host and proportion infected becomes significantly negative at passage number 9 ($p = 0.01$, M3.4) after being generally positive across the rest of the passages. This effect is mostly driven by the evolution genotype 17 lines, which have significantly higher specialization in productivity. Therefore,

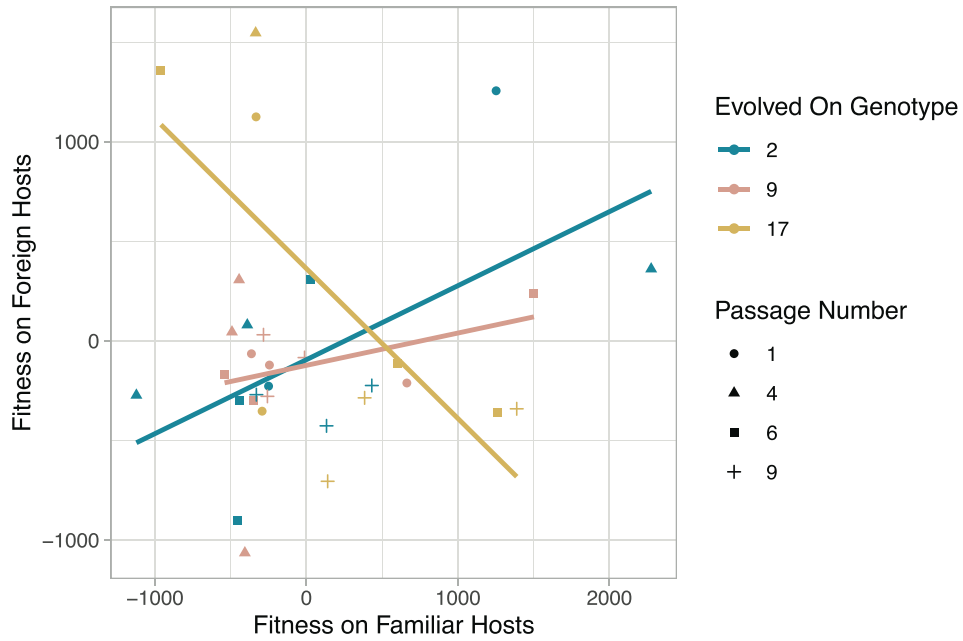

Figure 3. Correlation Between Fitness on Familiar and Foreign Hosts. Plot showing the correlation between each virus line's fitness on familiar and foreign hosts at passages 1, 4, 6, and 9. Effect sizes are taken from the GLMM models.

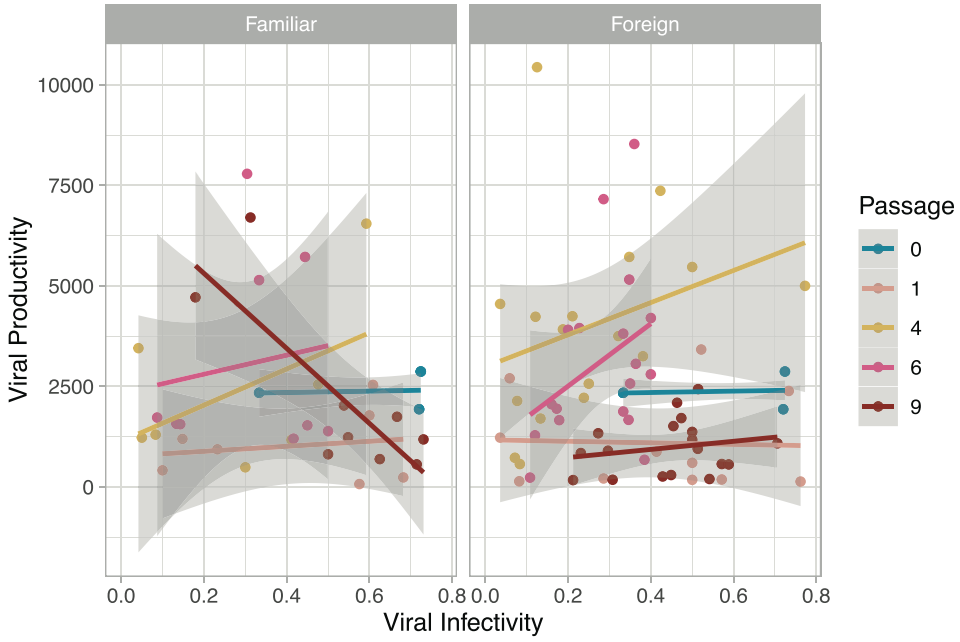

Figure 4. Relationship between Virus productivity and infectivity. Paneled plot showing the relationship between viral productivity and infectivity on both familiar (left panel) and foreign hosts (right panel) at each passage.

the direction of the relationship between viral productivity and infectivity changes from positive to negative depending on the passage number and whether the virus is infecting a familiar or foreign host (see Fig. 4). This indicates that productivity and infectivity are not strictly positively correlated traits and that specialism can evolve independently in either trait.

GENETIC VARIATION

Most variants are at low (<10%) frequencies, but there are several genomic regions that consistently have a high genetic variation (Fig. S10). These regions correspond with several ORFs homologous with genes in AcMNPV that have known functions including occluded virus production, oral infection, time to kill,

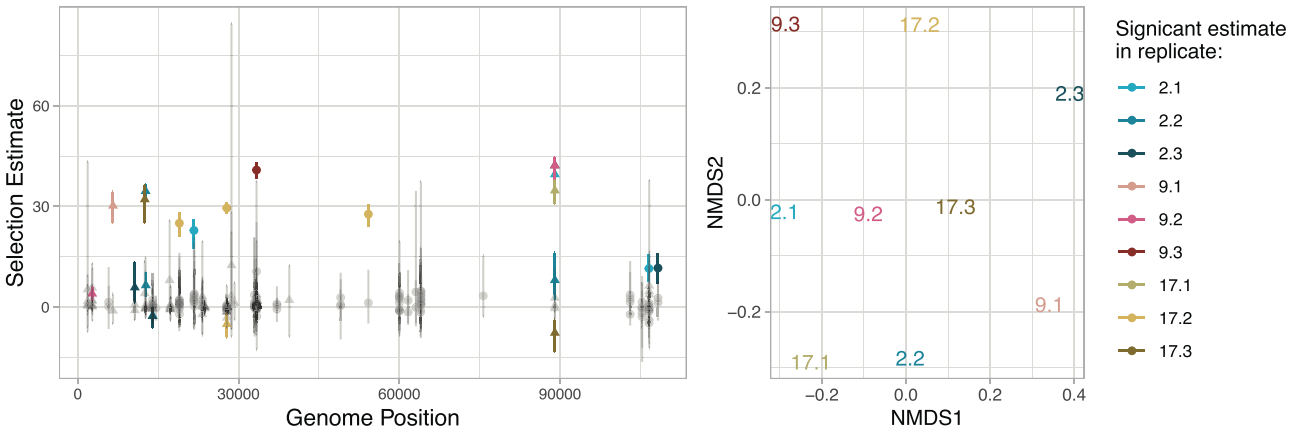

Figure 5. **(A) Genome-wide selection inferences for SNP and indel variants in each replicate. Significant variants (positive and negative) are colored by the replicate that they are detected in, and non-significant variants are light gray. Circle points represent SNPs and triangles represent Indels. See Figures S6–S8 for expanded versions of this figure. (B) Multidimensional scaling plots for passage 9 variant communities. Variant communities are transformed using the "canberra" method and plotted using "vegan." See Fig. S4 for "rare" and "common" variant NMDS plots.**

and host range (Table S4) (Harrison et al. 2016; Rohrmann 2019). We do not find that treatment significantly predicts variance in variant community composition at passage 9 in constrained ordination analyses with permutation tests (23% variance explained, $p = 0.69$), indicating that evolution genotype is not significantly predicting the frequencies of genetic variants (Fig. 5; Fig. S4).

Among the 18 alleles that were called as significant in the analysis of the $N_e = 92$ model (Fig. 5), we found three that were called as significant in two or more biological replicates from the same treatment (Table S5). Some putatively selected variants were shared across virus populations from two or more of the inbred lines, suggesting they may represent generalist adaptation to experimental conditions rather than adaptation to specific host genotypes (Fig. 5a; Figs. S6–S8). In general, we note that the inferred selection coefficients are mostly indicative of weak positive selection. If we suppose an effective population size of 92 (as in the demographic model we used for the selection inference), then the inferred values of 2Ns indicate per-allele effects ranging from 0.014 to 0.23.

Discussion

Specialization is critical to many of our theories of coevolution and the maintenance of diversity (Futuyma and Moreno 1988). In particular, specialization between parasites and their hosts is crucial for understanding patterns of disease emergence and spread (Woolhouse and Gowtage-Sequeria 2005). Here, we use experimental evolution techniques to test whether a granulosis virus can evolve to specialize on specific genotypes of its moth host. We find that the virus evolved to specialize in infectivity, productivity, and fitness on familiar host genotypes (Fig. 1).

A unique feature of our experiment is that we collect time series phenotypic and genetic data that allow us to explore the dynamics of specialization in novel ways. First, a key finding of our experiment is that the virus can evolve both higher viral infectivity and productivity on familiar host genotypes, thus specializing (Fig. 1). Several previous similar studies have also measured multiple fitness components related to specialization to find that pathogens could variably specialize on parasite virulence and/or transmission (Zhan et al. 2002; Nidelet and Kaltz 2007; Legros and Koella 2010; Kubinak et al. 2012). Kubinak et al. (2012) found that Friend complex virus evolved both higher viral productivity and virulence on familiar host genotypes and Zhan et al. (2002) found that fungal strains could specialize in both virulence and frequency, though this effect was inconsistent depending on the pathogen strain considered. However, Legros and Koella (2010) found that microsporidia specialized in infectivity, but not productivity, while Nidelet and Kaltz (2007) found that parasites specialized in growth assays, but not horizontal transmission. However, none of these previous studies have examined the correlations between their fitness components across time.

With our phenotypic time series data, we can see that the relationship between our two fitness components (infectivity and productivity) is positive at the start of the experiment but, by passage 9, evolves to be negative when infecting familiar hosts (Fig. 4). This correlation is likely to be an emergent property of selection where different virus lines are primarily selected to increase specialization by improving either viral productivity or viral infectivity, rather than an actual genetic trade-off between these traits. The likelihood of specializing on different fitness metrics may be related to evolution background as virus lines

evolved on host genotype 17 seem to be more specialized in their productivity at passage 9, while virus lines evolved on host genotype 2 seem to be more specialized in their infectivity at passage 9 (Fig. 1). This finding highlights the importance of measuring multiple fitness components when pathogen populations can use many strategies to increase their fitness.

Next, we can ask questions about the number of potential genes involved in specialism evolution. If specialization were to be driven by a few mutations of large effect, we would expect to see some degree of genetic parallelism in replicates and strong signatures of selection (if specialization is not driven by mutation accumulation). If there were many genetic options for specialization, we would not necessarily expect to see the phenotypic parallelism of the experiment reflected at the genetic level and selection on any one variant would be weaker.

In our experiment, the evidence indicates that specialization was driven by many variants of small effect (Fig. 5). We did not observe any clear signals of selective sweeps where low-frequency alleles swept to high frequency. Given the relatively high depth of coverage of our samples and the quality of the sequencing data, it is unlikely that we failed to detect many (if any) sweeps. Furthermore, our selection analysis does not identify any variants with strong parallel signatures of selection across replicates (Fig. 5). These results are likely influenced by the facts that our starting population is genetically diverse, so our experiment is more likely to select on standing variation (Long et al. 2015), and that we serially passage through hosts, so transmission bottlenecks likely genetically bottleneck our lineages (Kennedy and Dwyer 2018). It is also possible that selection for specialization may have been obscured by the initial selection for generally improved fitness in experimental conditions, though we did not see strong, parallel signatures of selection for either general or specialist fitness.

Our results finding many candidate genes with lower selection coefficients are generally in line with previous evolve and re-sequence experiments that start with standing genetic variation and less-specific environmental stressors, though clonal interference may have been less prominent in our experiment due to the relatively smaller bottleneck sizes in vivo infection processes (Miller et al. 2011; Tenaillon et al. 2012; Lang et al. 2013; Long et al. 2015; Schlötterer et al. 2015). In the context of virus adaptation to host genotype, Middlebrook et al. (2021) also do not see parallel genetic evolution when FVC virus specializes on mice with different MHC genotypes from a clonal starting population, although they did see evidence that virus populations adapted to each MHC genotype are more similar to each other than to those adapted to foreign MHC types.

Second, we can ask questions about whether specialization is driven by antagonistic pleiotropy, conditionally positive adaptation resulting in fitness asymmetries, or mutation accumulation in alternate environments. If specialization were to be driven by antagonistic pleiotropy, we would expect to see that the most fit replicates on the familiar host are the least fit on the foreign host and that positive selection acts on variants. We would not have clear predictions for how total fitness across all the genotypes would change over time as this would depend on the symmetry of the trade-off shape. In the case of conditionally positive alleles resulting in fitness asymmetries between familiar and foreign hosts, we would expect to see slightly positive or neutral fitness correlations between familiar and foreign hosts, positive selection on variants, and overall increases in total fitness across all the genotypes. In the case of mutation accumulation, we would expect negative fitness correlations between familiar and foreign hosts (the most specialized are those that are worst on foreign hosts), no evidence of positive selection since MA is driven by drift, and overall decreases in total fitness across all the genotypes.

Of course, these mechanisms are not exclusionary, especially in our case where many variants can affect specialization. These predictions may therefore be muddied if multiple mechanisms are driving specialization. Additionally, any directional fitness changes to overall experimental conditions might hamper our ability to fully assess whether fitness correlations between genotypes are positive or negative (as some replicates may just be the "most adapted" to the general environment) and our ability to assess changes in total fitness across genotypes in the system.

We find that correlations between fitness on familiar and foreign hosts significantly vary depending on the evolutionary history of the virus (Fig. 3). There is a negative correlation between fitness on host genotype 17 and foreign genotypes, suggesting that specialization on this host could be consistent with any mechanism. However, correlations between fitness on familiar and foreign host genotypes are positive for virus specializing on host genotypes 2 and not significant for virus specializing on host genotype 9. This suggests specialization driven by asymmetric conditional positivity. Therefore, it is likely that multiple mechanisms contribute to specialization in our system.

From our sequence analysis, we do not see evidence of strong, parallel positive selection on any variants. We observe many instances of subtle frequency differentiation during the course of the experiment, which seems a likely candidate to explain the genetic mechanism for adaptation (Fig. 5; Figures S6–S8). Thus, the sequencing data cannot help to exclude potential specialization mechanisms as it is unclear whether these weakly positively selected alleles collectively have strong enough effects to explain phenotypic specialization (as would be predicted by positive selection on antagonistically pleiotropic or conditionally positive alleles) or whether additional drift-based mutation accumulation processes are also needed to explain the specialization in our system.

Finally, the total fitness of virus lineages across all host genotypes does not increase continuously though the experiment (Fig. 2). Total fitness does increases from passage 1 to passage 4 but plateaus from passage 6 to passage 9, which is also when we see our largest changes in specialization. This would suggest that antagonistic pleiotropy or a balance of conditional positivity and mutation accumulation is driving specialization. It also suggests that PiGV quickly reached a point of being fairly well adapted to experimental conditions so that directional selection to overall experimental conditions is less likely to obscure patterns resulting from specialization. However, a caveat to these trends in total fitness is that our assay scheme was designed to best test the changes in relative fitness on different genotypes over time and so assayed viruses from different passages on different days. Therefore, these trends in total fitness (but not relative fitness) might be confounded by random day effects.

In this experiment, we have shown that *Plodia interpunctella* granulosis virus can evolve to specialize on specific genotypes of its host and that specialization is not driven by strong selection on a few alleles. However, we cannot precisely determine the evolutionary mechanism of this specialization. Putting our evidence together, it seems most likely that the evolution of specialization in our experiment is driven by many genetic variants and by multiple mechanisms. For lines evolved on host genotype 17, which also showed the most specialization via viral productivity, specialization may be most parsimoniously explained by antagonistic pleiotropy as this would explain their negative fitness correlations with overall stable fitness. For lines evolved on genotypes 2 and 9, specialization may be most parsimoniously explained by a combination of weakly positive fitness asymmetries and mutation accumulation in alternate environments as these mechanisms could have collectively driven specialization while their opposing effects on total fitness would result in no total fitness changes. The weak signatures of selection and lack of genetic parallelism in our sequence analysis would fit with these hypotheses if antagonistic pleiotropy and conditionally positive fitness asymmetries are driven by many variants of small effect.

Of course, the findings of our experiment may be limited in their universality as the *Plodia intepunctella* and PiGV system is but one model system with unique biological features like obligate killing and, while our serial passaging protocol closely mimics the natural transmission pathway of oral ingestion of virus killed cadavers, it is not exactly natural transmission in that we homogenize cadavers and transmission is constrained to happen on a certain day after exposure (day 20), to a specific larval instar (third), and at a specific dose. Thus, further studies on this topic in different model systems will only help to strengthen our understandings of the dynamics of specialization.

In conclusion, we used an experimental evolution approach to determine whether a baculovirus could evolve to specialize in specific genotypes of its moth host. We find that virus does evolve higher infectivity, productivity, and fitness on familiar host genotypes (Fig. 1). This specialization may be variably driven by combinations of antagonistic pleiotropy, conditionally positive alleles leading to fitness asymmetries, and mutational accumulation on foreign host genotypes in our different evolutionary conditions. Time series data show that specialization in fitness evolves over the time course of the experiment and that the different fitness components of virus lineages may be independently selected (Figs. 2 and 4; Fig. S2). Our results demonstrate that gene-by-gene interactions are evolvable in the *Plodia interpunctella* and PiGV model system and suggest that the system has promise for experiments on the ecological conditions that shape selection on specialization and niche breadth.

AUTHOR CONTRIBUTIONS

E.V. and M.B. designed the experiment. E.V., N.D., A.Y., and D.A. collected data. E.V., L.B., and L.U. analyzed the data. E.V., L.B., L.U., and M.B. wrote this manuscript.

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DATA ARCHIVING

All data presented in this study are available in the supplementary materials, alongside an annotated R script used for analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary information
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